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# DAILY RHYTHMS IN CONCENTRATION OF PLASMA CORTISOL IN MALE AND FEMALE GULF KILLIFISH, *FUNDULUS GRANDIS* [1]

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Daily rhythms of adrenal corticoids have been implicated recently in the regulation of fat stores in vertebrates (review, Meier, 1972). In intact *Fundulus grandis* maintained in continuous light, injections of cortisol entrain a 24-hour rhythm of fattening responses to mammalian prolactin (Meier, Trobec, Joseph and John, 1971). Daily injections of prolactin given 18 or 24 hours after daily injections of cortisol promote increases in fat stores whereas injections of prolactin given 6 or 12 hours after cortisol injections cause decreases in body fat.

Although considerable information regarding the daily rhythm of concentration of plasma adrenal corticoids is available for mammals, the possible existence of such rhythms in fish has received little attention. Daily rhythms of plasma adrenal corticoids have been reported in the male channel catfish, *Ictalurus punctatus* (Boehlke, Church, Tiemeri and Eleftheriou, 1966), and a daily variation in plasma cortisol has been reported in the male *Fundulus grandis* investigated during an 8-hour portion of the day (Srivastava and Meier, 1972). Both studies of fish and most of the studies of mammals and birds were carried out with males only under carefully controlled conditions in the laboratory. Because various stresses may be expected to stimulate increases in plasma concentrations of adrenal corticoids in vertebrates, including fish (Donaldson and McBridge, 1967), the possibility that the rhythms of adrenal corticoids have important physiological roles depends on whether the rhythms exist under natural conditions. This study was performed with *Fundulus grandis* to determine the concentrations of plasma cortisol throughout the day in male and female fish taken from a brackish lake.

## Materials and Methods

Blood, gonads and intact carcasses of male and female specimens of *Fundulus grandis* were collected during June and August, 1971. The study site is a deep thirty-six acre brackish lake about 80 miles south of Baton Rouge, Louisiana. During both months of the study the salinity was 115 milliosmoles. Surface temperatures ranged from a low of 29° C at dawn to a high of 31° C nine hours after dawn in June and in August. The temperature on the bottom near the shore at a depth of 0.5 meters was a constant 29° C in both months. Sunrise and sunset on June 6, the first collection date, were at 0502 and 1905 Central Standard Time, respectively. On August 8, sunrise and sunset were at 0527 and 1853 C.S.T.

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Daylength on June 6 is longer by 37 minutes than on August 8. The moon was in the full phase in both months of the study.

Specimens of *Fundulus grandis* weighing 8–20 grams were collected by seining five days prior to the date of exsanguination and distributed equally by number and sex among six hardware cloth holding cages. These cages were positioned in the shallow (depth 0.5 m) water near the shore where the fish are generally found. Aquatic vegetation of the type frequented by *Fundulus grandis* was placed in each cage to provide a refuge for the subordinate individuals. The cages were watched continuously and were not disturbed during the waiting period. During the days prior to exsanguination the fish in the cages exhibited behavior similar to the uncaged fish in the area, including feeding, schooling, and (in males) aggressive display.

Collection of blood was conducted at one hour after dawn (30 minutes after sunrise) and at every fourth hour thereafter for the next twenty hours. At each time of collection, a single cage was removed from the water and the fish were placed in an anesthetizing solution of tricaine methane sulfonate (Sigma) having a concentration of one gram per liter. The fish were immobilized within thirty seconds. Blood was collected directly from the heart with a heparinized capillary tube. Three workers were able to complete the exsanguination of a group of up to fifteen fish well within fifteen minutes. Blood from each fish was labelled according to the fish's order in the sampling sequence, so that a possible change in circulating levels of cortisol initiated by the disturbance could be determined. A single fish usually yielded sufficient blood for three 10 $\mu$l samples of plasma. Following the collection, the blood was temporarily stored on ice at the study site. There were 27 blood samples in June and 39 in August that were taken from males, and 30 samples in June and 44 in August that were taken from females. Except for the samples taken from the males in June, the numbers of samples were approximately equal for each of the 6 daily sampling times. The blood specimens taken at 0200 from males in June were accidentally lost.

Upon arrival at the laboratory, the blood was centrifuged and the plasma was separated from the packed cells and stored by freezing. Plasma adrenal steroids are stable for many months if kept frozen (Guillemin, Clayton, Lipscomb, and Smith, 1959). Plasma concentrations of cortisol, the major corticosteroid in poeciliid fishes (Chester Jones, Chan, Henderson and Ball, 1969) were determined using the competitive protein-binding radio-assay of Murphy (1967) using human plasma as the source of CBG and Florisil as adsorbent. For each assay, one sample from each time group was taken in triplicate. Tritium counting was done with a Beckman Liquid System using toluene scintillation solution containing 2,5-diphenyloxazole (0.3% w/v), 1,4-bis-2(5 phenyloxalyl)-benzene (0.01% w/v) and Triton-X100 (2:1 by volume). To test whether other plasma steroids in *Fundulus grandis* might interfere prohibitively, plasma cortisol was measured directly and after separation by thin layer chromatography. In male specimens of *Fundulus grandis* maintained indoors on a 12-hour photoperiod, the results were comparable for blood collected at 1, 5, and 9 hours after the onset of light.

The gonadal weights and fat stores were determined in 20 randomly selected fish in June and again in August. The gonads were preserved immediately in ethanol and weighed after several days. The gonadal weight is expressed as a percentage of the fresh weights of the fish (gonadosomatic index). The total body lipid was determined by Soxhlet extraction with ether and is expressed as a percentage of the dry body weight (dry lipid index).

## Results

Gonadal regression similar to that observed by Matthews (1939) in *Fundulus heteroclitus* occurred in both sexes of *Fundulus grandis* between June and August (Fig. 1). The gonadosomatic index in males was 0.36 ± 0.03 in June and 0.24 ±

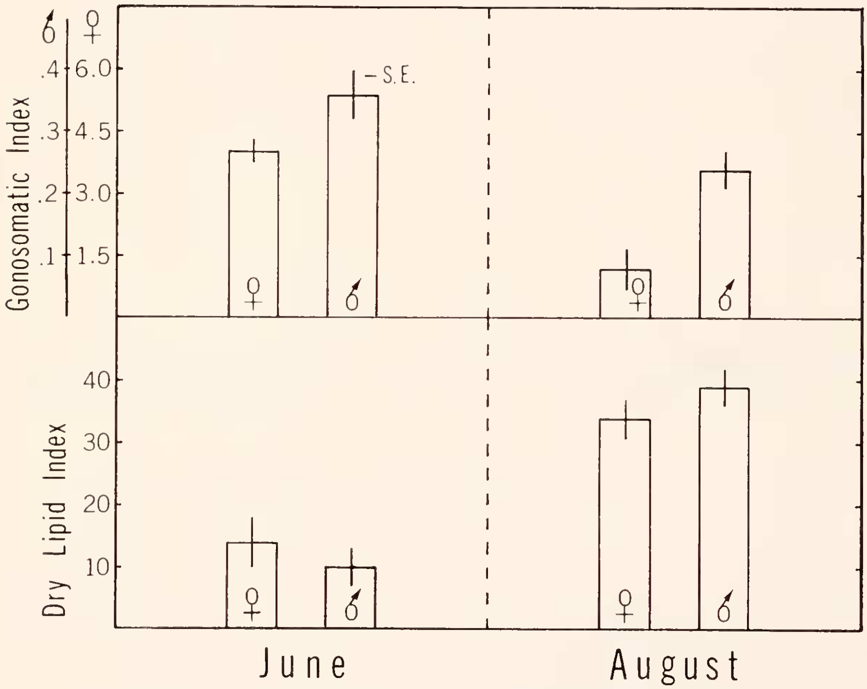


FIGURE 1. Gonadosomatic and dry lipid indexes in June and July.

0.03 in August. In females, the gonadosomatic index was 4.31 ± 0.23 in June and 1.43 ± 0.36 in August.

The regression of the reproductive system between June and August is accompanied in both sexes by an increase in fat stores (Fig. 1). The dry lipid index rises from 12.10 ± 0.39 in June to 34.75 ± 4.0 in August. There is no significant difference in fat content between the sexes in either month, and there is no correlation between dry lipid index and whole body weight in either month.

The plasma cortisol content (p.c.c.) of all the fish averaged 21.9 $\mu g\%$ in June and 23.2 $\mu g\%$ in August. These overall seasonal means do not differ significantly. The overall means of the sexes are the same (males: 22.5 ± 1.3; females: 22.5 ± 1.1). However, there are marked variations in the p.c.c. of both male and female *Fundulus grandis* during a twenty-four hour period (Figs. 2 and 3). According

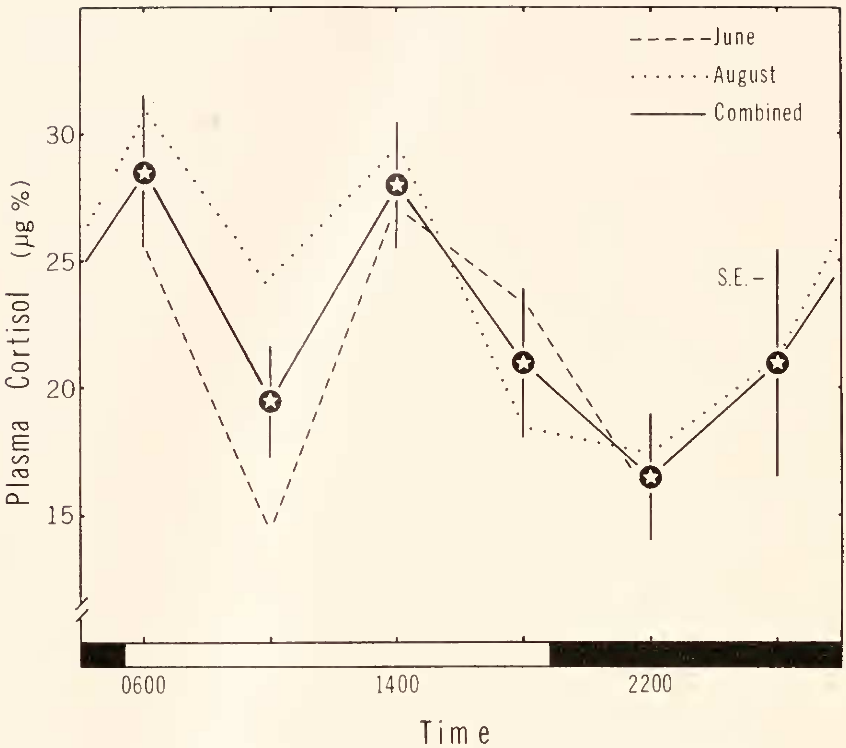


FIGURE 2. Daily rhythms in concentration of plasma cortisol in male specimens of *Fundulus grandis* in June and August.

to a least-squares analysis of variance (Snedecor and Cochran, 1967), the daily variations of the combined data of June and August are significant ($P < 0.05$) for each sex. In addition, the rhythm of each sex is distinct.

According to orthogonal comparisons (Snedecor and Cochran, 1967), the daily rhythm of cortisol in males has linear ($P < 0.05$) and quartic ($P < 0.01$) relationships, indicating that although there is a general downward trend in the cortisol levels in the twenty hours following dawn (linear trend), the rapid rise observed nine hours after dawn at 1400 is also significant (quartic trend). Thus, the daily cortisol rhythm in males in both June and August is bimodal, with peak values occurring near dawn (0600) and eight hours later.

The daily rhythm of plasma cortisol in female *Fundulus grandis* for June and August combined differs from that in the males in that it bears cubic relationships ($P < 0.05$), indicating the presence within each day of a single alternation between periods of high and low concentrations. The smaller sample size in June is unfortunate in that it may have prevented statistical verification of a possible seasonal change in the time of the daily rise of p.c.c. in female fish. During both months, the peak of plasma cortisol occurs at dawn, followed by a rapid fall within four hours, similar to that occurring in males. In the females, however, there is only one daily

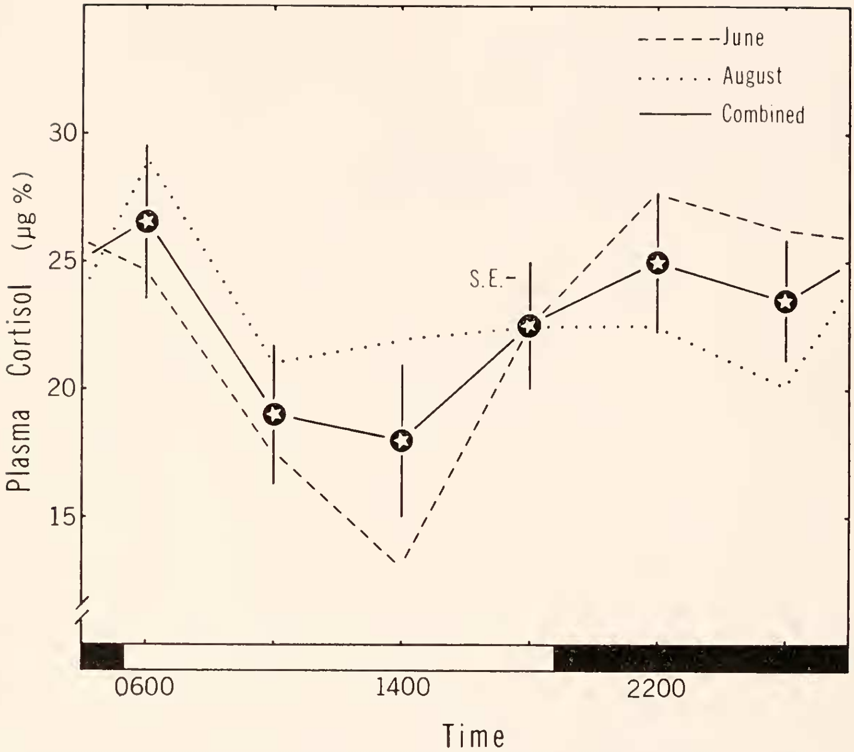


FIGURE 3. Daily rhythms in concentration of plasma cortisol in female specimens of *Fundulus grandis* in June and August.

rise in p.c.c. occurring between 1400 and dusk in June and between 0200 and 0600 in August. High plateau levels of cortisol were maintained throughout the night in June.

The disturbance associated with the sampling procedure caused a significant rise in cortisol content within a period of less than fifteen minutes. For the entire group of fish employed in the study, the average increment in p.c.c. for each unit increase in sample sequence number was 0.72 μg%. The regression coefficient (0.72) is significantly different from zero according to analysis of variance of linear regression. The rise in p.c.c. by the end of a fifteen minute series of exsanguinations was about 7 μg%. The data do not permit any conclusions concerning possible changes in p.c.c. occurring in the time interval (30–60 seconds) between the initial disturbance and the first exsanguination.

## DISCUSSION

The existence of daily rhythms of plasma cortisol in *Fundulus grandis* is not surprising in view of the widespread occurrence of daily rhythms of adrenocortical hormones in the higher vertebrates. The presence of these rhythms under natural conditions in a fish strengthens the idea that they have important functions.

Temporal relationships between the daily rhythms of prolactin and cortisol have been implicated in the control of fat stores. Prolactin stimulates fat deposition in *Fundulus chrysotus* (Lee and Meier, 1967), *Fundulus kansae* (Mehrle and Fleming, 1970), *Fundulus grandis* (Joseph and Meier, 1971), *Cyprinodon variegatus* and *Fundulus similis* (de Vlaming and Sage, 1972). In all of these investigations, however, the time of injection with respect to the photoperiod proved to be of fundamental importance in determining whether prolactin elicited gains or losses in fat stores. These variations in response to prolactin appear to be the result of daily rhythms of cortisol. In specimens of *Fundulus grandis* maintained in continuous light, daily injections of cortisol entrain daily rhythms of fattening responses to prolactin (Meier, Trobec, Joseph, and John, 1971). That is, daily injections of prolactin given at 6 or 12 hours after injections of cortisol cause losses of fat stores, whereas prolactin injections administered at 18 or 24 hours after cortisol cause increases in fat.

One can derive some interesting temporal correlations by comparing our findings of the daily rhythms of concentration of plasma cortisol with previous studies of *Fundulus grandis* which involve daily rhythms of fattening responses to prolactin entrained by daily photoperiods (Joseph and Meier, 1971) and by daily injections of cortisol (Meier, Trobec, Joseph, and John, 1971). The fattening response to prolactin occurs at 4 to 8 hours after dawn in fish maintained on 8-, 12-, or 16-hour daily photoperiods, and at 18 to 24 hours after daily injections of cortisol in fish maintained in continuous light. In order for cortisol to account for the fattening response to prolactin at 4 to 8 hours after dawn, one would predict on the basis of these findings that the daily rise of plasma concentrations of cortisol should occur about 6 to 12 hours after dawn. This prediction agrees well with our findings in that the first daily increase in plasma cortisol in males occurred about 9 hours after dawn and the daily increase in females occurred about 12 hours after dawn. Inasmuch as the cortisol rhythm is bimodal in the male with a second increase in concentration at 20 to 24 hours after dawn, one would anticipate that another period of fattening response to prolactin might be found in males during the dark, 20 to 22 hours after dawn. Injections at that time of day have not as yet been done in *Fundulus grandis*. For a graphic description of the temporal relations, see Figure 4.

The central role of cortisol in mediating the photoperiodic entrainment of the daily fattening response to prolactin in *Fundulus grandis* is similar to the roles ascribed to adrenal corticoids in the photoperiodic entrainment of other daily responses to prolactin: fattening responses in lizards, birds and mammals; cropsac responses in pigeons; red eft water drive responses in the spotted newt; reproductive photosensitivity and photorefractory responses in sparrows; and migratory restlessness and orientation responses in a migratory bird (reviews, Meier and MacGregor, 1972; Meier, 1972). The close correlation of many of the results obtained by injection of hormones with assays of the hormones (similar to that described in Fig. 4) provides strong evidence that the interrenal system has an important role in photoperiodic entrainment of daily rhythms.

So far as we can ascertain, a sexual difference in the daily rhythm of adrenal corticoids has not been reported in any other vertebrate. It should be noted, though, that the possibility has seldom been taken into account. A comparison of

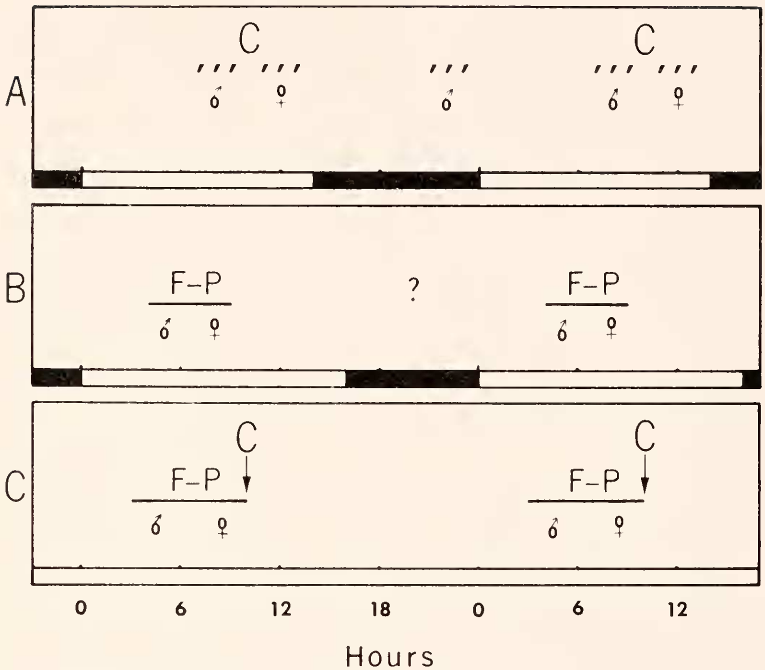


FIGURE 4. Temporal relations of the daily rhythm of cortisol in the photoperiodic entrainment of the daily fattening response to prolactin; (A.) the times of the daily increases in concentrations of plasma cortisol (C) in *F. grandis* outdoor (reported herein); (B.) the time of the daily fattening response to prolactin (F-P) in *F. grandis* maintained on a 16-hour daily photoperiod. Tests were not made during the hours of darkness (Joseph and Meier, 1971); (C.) the temporal relation between daily injections of cortisol and the fattening responses to prolactin in *F. grandis* maintained in continuous light, (Meier, Trobec, Joseph, and John, 1971).

the daily rhythms of the sexes (Figs. 1 and 2) suggests that some factor is influencing the daily rhythm in one sex and not in the other. The most conspicuous difference is the sharp rise in plasma cortisol at 9 hours after dawn followed by a sharp drop at 13 hours after dawn in the male, but not in the female. The possibility that the midday peak in males is a result of interference from gonadal steroids (Murphy, 1967) has been eliminated because this peak is also present after separation of cortisol by thin layer chromatography (see Materials and Methods). In addition, the peak is also present in hypophysectomized *Fundulus grandis* in which the gonadal weights are very small (Srivastava and Meier, 1972). The physiological basis and significance of the sexual difference in the daily rhythm of cortisol remains to be determined.

Because the interrenal gland of all vertebrates is stimulated by adrenocorticotropic hormone (ACTH), it has seemed reasonable to assume that a daily rhythm in concentration of plasma corticoids would be a direct result of a daily rhythm of release of pituitary ACTH. However, when this assumption was tested in hypophysectomized male *Fundulus grandis,* it was discovered that the daily rhythm of plasma cortisol was essentially the same as in intact fish (Srivastava and Meier, 1972). In addition, the phase of the rhythm may be inverted in both hypophysectomized and intact fish by inverting the 12-hour daily photoperiod. Thus, the existence of the daily rhythm of plasma cortisol as well as the photoperiodic entrainment of the rhythm does not depend on a daily rhythm of pituitary ACTH. It might be expected that the discovery of the mechanism involved in regulating the daily rhythm of plasma cortisol will considerably increase our understanding of endocrinology and biological rhythms. Apparently, cholinergic systems are involved (Meier and Srivastava, unpublished).

The increase in concentration of plasma cortisol after immobilization with tricaine methane sulfonate is in keeping with what is expected in animals after an applied stress. The increase of 7 μg% in fifteen minutes corresponds with an increase of 5.3 μg% (from 8.1 to 13.4 μg%) in *Salmo gairdneri* following half an hour of forced activity in very shallow water (Donaldson and McBride, 1967). An explosive increase in concentration of plasma cortisol (from 6.2 μg% to 11 μg%) has been reported to occur in *Carassius auratus* within 15 seconds of an osmotic challenge with 0.1% NaCl (Singley and Chavin, 1971). However, a similar sharp increase in plasma cortisol following handling disturbances does not occur in *Carassius auratus* (R. E. Spieler, Louisiana State University, unpublished), nor in *Fundulus grandis* (Srivastava and Meier, 1972). The general pattern of change in concentration of plasma adrenal corticoids following a single applied stress in most animals investigated involves a gradual increase in concentration reaching a maximum in 15–30 minutes followed by a decline to normal levels usually within one to two hours.

The increase in plasma concentrations of cortisol following stress might be expected to interfere with any functions controlled by a daily rhythm of plasma cortisol. Recent studies in our laboratory indicate that such is the case in a number of vertebrate species including *Fundulus chrysotus* (Meier, Trobec, Haymaker, MacGregor and Russo, 1973). Disturbances of handling at specific times of day repeated daily for 10 days caused substantial increases or decreases in fat stores depending on the time of day when the disturbances were made. The reproductive system was also stimulated or inhibited depending on the time of disturbance. Under the conditions of the present study which approach natural conditions, whatever disturbances that occur do not obscure the daily rhythm of cortisol. However, appropriate stresses of sufficient intensity and/or duration may be expected to interfere with the daily rhythm of cortisol and with the functions or conditions that are controlled by the rhythm.

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## Summary

Daily rhythms of plasma cortisol were found in male and female *Fundulus grandis* examined in a brackish lake in June and August. The rhythm differed in

the two sexes. The rhythm of the males was bimodal; the peaks occurred at one hour and at nine hours after sunrise. The rhythm of the females was unimodal with high concentrations late in the day and at night until one hour after sunrise.

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